

#10
CJW
PATENT
Our Docket: SALK2190 6/23/99

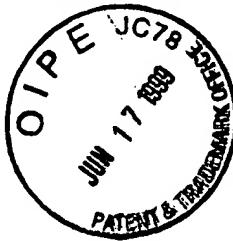
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
O'Gorman et al.

Serial No.: 08/919,501

Filed: August 28, 1997

For: SITE-SPECIFIC RECOMBINATION
IN EUKARYOTES AND
CONSTRUCTS USEFUL
THEREFOR



) Group Art Unit: 1633

) Examiner: M. Wilson

CERTIFICATION UNDER 37 CFR §1.8

I hereby certify that the documents referred to as enclosed herein are being deposited with the United States Postal Service as first class mail on this date, 6/14, 1999 in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Stephen E. Reiter, Reg. No. 31,192
(Name of person mailing paper)

Signature

Date

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF
APPLICANT UNDER 37 C.F.R. §131

Sir:

We, Stephen O'Gorman and Geoffrey Wahl, the joint inventors of the above-identified application, do hereby declare and state that:

We are familiar with the content of the above-identified application.

We are aware that claims 1, 2, and 4 of the above-identified application have been rejected for alleged anticipation under 35 U.S.C. §102 over Lewandoski *et al.* (*Current Biology* 7:148-151, 1997) (hereinafter "Lewandoski") and claims 1-2, 4-5, 10-16, 18-19 and 24-44 have been rejected for alleged obviousness under 35 U.S.C. §103(a) over a combination of references including Lewandoski.

Best Available Copy

The claimed invention was conceived and reduced to actual practice by us in the United States as joint inventors prior to the publication date of the Lewandoski article, as supported by the evidence which follows:

At the time the present invention was conceived and reduced to actual practice, Stephen O'Gorman was the leader of the research team that performed the experiments set forth as Examples 1 through 5 of the above patent application, which experiments were completed in laboratories of The Salk Institute for Biological Studies in La Jolla, California prior to the publication date of the Lewandoski article. In support of this statement are attached true copies (with only the dates redacted) of pages from laboratory notebooks belonging to The Salk Institute for Biological Studies, as follows:

1. A 652 bp fragment of the mP1 promoter was isolated by PCR using genomic DNA from CCE embryonic stem cells as a template (Lab notebook pages 1 through 5).
2. The mP1 promoter fragment was fused to a modified Cre coding sequence in the plasmid pOG304M (Lab notebook pages 6 through 10).
3. A Cre expression plasmid pOG231 was prepared by fusing a modified Cre coding sequence to a synthetic intron and CMV promoter. (Lab notebook pages 11 through 13).
4. A plasmid pOG277 containing a loxP-flanked neomycin cassette was prepared by inserting a wild-type loxP site into pBSKS (Stratagene) and then cloning a neomycin cassette between interations of this loxP site. (Lab notebook pages 14 through 16).
5. A hoxb-1 targeting construct containing a NruI site was prepared and the loxP-flanked neo cassette from pOG277 was inserted into the NruI site. (Lab notebook pages 17 through 29).

6. The P2Bc allele of Figure 1 was created by inserting a Lox-P flanked neomycin cassette and a β-GAL sequence into RNA polymerase II. (Lab notebook pages 45 through 61).
7. Transgenic mice were obtained from fertilized mouse oocytes injected with the protamine-Cre fusion gene from pOG304M (Lab notebook pages 62 through 65).
8. Heterozygous ProCre males were mated to wild-type females and the resulting progeny were examined by Southern blotting to determine the segregation pattern of ProCre nucleic acid constructs and P2Br alleles. (Lab notebook pages 30 through 44 and 62 through 67).

Thus, we maintain that the subject matter contained in the above-identified application was conceived and reduced to actual practice by us prior to the date of publication of the Lewandoski reference.

We further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

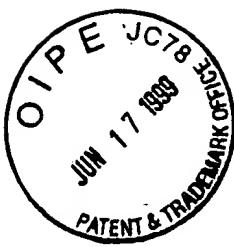
6/14/99
Date

Stephen O'Gorman
Stephen O'Gorman

6/14/99
Date

Geoff Wahl
Geoffrey Wahl

Attachments: Laboratory Notebook Pages 1-67.



Protamine promoter PCR

FILENAME: PROTAMINE PROMOTER PRIMERS
IN: LAB/SEQUENCES/CRE/PROTAMINE

This is a pair of primers to amplify the mouse protamine 1 promoter from -560 to the translational start. The 5' primer includes a Bgl II site. The 3' primer contains a SmaI site. Both of these sites are meant to allow us to drop the PCR product into pOG234. The PCR product should be about 570 bp in length.

Primer positions: U 1 L 630

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol
 5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3' → GMW 310
 3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCATC 5'

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'
 3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCA

Upper/Lower: the most stable dimer overall: 5 bp, -9.4 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'
 3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCATC 5'

Lower Primer, 38-mer [630]:

Lower Primer: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' CTACCCGGGTGCTGGCTTGGCCGGAGCTGGCTCAGAG 3' → GMW 311
 3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGG

Lower Primer: the most stable dimer overall: 8 bp, -18.6 kcal/mol

5' CTACCCGGGTGCTGGCTTGGCCGGAGCTGGCTCAGA
 3' GAGACTCGGTCGAGGGCCGGTTCGGTCGTGGGCCATC 5'

Hairpin: $\Delta G = -8.0$ kcal/mol, Loop = 12 nt, Tm = 98°

5' CTACCCGGGTGCTG
 3' GAGACTCGGTCGAGGGCCGGTTC

Protamine promoter PCR

Upper Primer, 37-mer [1]:

Upper Primer: the most stable 3'-dimer: 2 bp, -1.3 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAGT 3'

3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Upper Primer: the most stable dimer overall: 6 bp, -7.9 kcal/mol

5' GAAGATCTGTCTAGTAATGTCCAACACCTCCCTCAG

3' TGACTCCCTCCACAACCTGTAATGATCTGTCTAGAAG 5'

Hairpin: $\Delta G = 1.3$ kcal/mol, Loop = 4 nt

5' GAAGATC

3' TGACTCCCTCCACAACCTGTAATGATCTGT

Optimal Annealing Temperature: 61.3°

Product length, GC content & Tm: 667 bp, 52.5% GC, 80.4°

Product Tm - Upper Primer Tm: 13.7°

Primers Tm difference: 17.5°

Upper Primer: 37-mer, pos. 1, Tm = 66.7°, 3'-pentamer $\Delta G = -6.4$ kcal/mol

Lower Primer: 38-mer, pos. 630, Tm = 84.1°, 3'-pentamer $\Delta G = -6.7$ kcal/mol

Salt & DNA Concentrations (fixed in this option): 50 mM and 250 pM, respectively

NOFILE

this is a sequencing primer for the protamine promoter. It is from the top strand of the promoter starting at 130 bp from the protamine translation Initiation codon and extending for 20 bp.

It is oligo 319

Current Oligo, 20-mer [534]:

Current+ Oligo, the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' GAGGAAGAGGGTGCTGGCTC 3'
 |||
3' CTCGGTCGTGGAGAAGGAG 5'

Current- Oligo: the most stable 3'-dimer: 3 bp, -3.2 kcal/mol

5' GAGCCAGCACCCCTCTTCTC 3'
 ||| || ||| |||
3' CTCCCTCTCCCACGACCGAG 5'

Current+ Oligo: the most stable dimer overall: 3 bp, -3.2 kcal/mol

5' GAGGAAGAGGGTGCTGGCTC 3'
 |||
3' CTCGGTCGTGGAGAAGGAG 5'

Hairpin: $\Delta G = 0.9$ kcal/mol, Loop = 8 nt

5' GAGGAAGAGGGTG
 |||
3' CTCGGTC

PCR REACTIONS TO PULL OUT PROTHAMINE PROMOTER

Primer mix 310
311

Resuspend in 200 μl TE
Read 260/280 at 1:100 dilution
Concentration = 2 × A₂₆₀

	A320	A280	A260	280/260	260/280	
Primer mix	0.0047	0.5788	1.0482	0.5501	1.8177	310
	0.0090	0.7395	1.3386	0.5494	1.8202	311

$$310: 10\lambda \div 2.1 \text{ g/\lambda} = 4.8 \lambda$$

$$311: 10\lambda \div 2.7 \text{ g/\lambda} = 3.7 \lambda$$

$$9.5 \lambda \text{ H}_2\text{O to 1\% = 100\lambda}$$

Reactions

Want to use ~ 18 μg DNA/reaction; have stock that is 0.66 g/g

Want to run 8 reactions

Mix for 10 μg reactions

- 15 μg DNA

235 μl TE

Boil DNA 5 minutes

Place on ice immediately

THESE REACTIONS WERE USED IN THE CONSTRUCTION
OF "PLASMIDS 304 AND 305"

GOOD PRODUCT OBTAINED (SEE NEXT PAGE)

PCR Reactions to Clone Promoter Primes

#	DNA	MgCl ₂	NTP	50mM Tris-HCl	Primes	H ₂ O	2X PCR
1	25 μ	2	2		3	20	50 μ
2		2	2		5	18	
3		2	2		3	16	
4		1	1		3	14	
5		3	3		3	12	
6		4	4		3	10	
7		5	5		0	8	
8		3	3				

- ① Iced tubes and place on 1°Q
- ② Add 2X PCR
- DNA
- MgCl₂ NTP
- Primes
- ③ Add 0.5 μ Tag polymerase
- ④ Mix by trituration.

Cycling Parameters

1x 94°C, 60 sec

35x 92°C, 30 sec
55°C, 1 min
72°C, 72°C

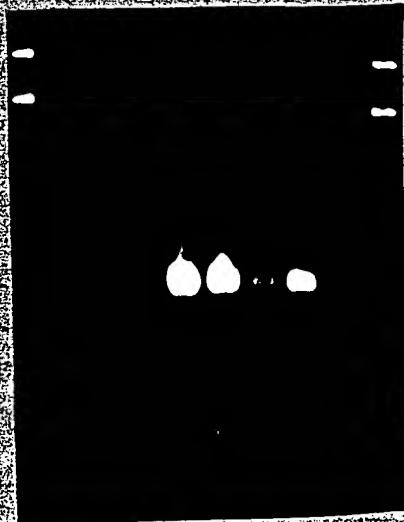
5/12/95 10 μ each reaction run on a standard minigel.

#9 = blank



Row 9 placed by the technician
amounts of product in previous
reactions. There is no good reason
why #4 should show lots of
product and lanes 1-9 should
show none.

It is possible that #4 should
be the clearest reaction (with
MgCl₂)



1
5

304
305

Subclone Prostaglandin Promoter.

① Clean up and ppt PCR reaction #4, product.

- add 120 μ l TE
- extract with 200 μ l P/C
- add 20 μ l 3M NaOAc
- add 1 μ g glycogen
- add 300 μ l EtOH
- 15 minutes ice
- 30 minutes 1014Y
- wash pellet 70%
- resuspend in 20 μ l

② Digest PCR product with $B\beta$ II and $P\alpha$ A \bar{I}

PCR #4 only.

20 μ l DNA
20 μ l 10X #3
10 μ l $P\alpha$ A \bar{I}
10 μ l $B\beta$ II
140 μ l H₂O

③ Digest pCG234 with $B\beta$ II and $S\alpha$ I

7.5 μ l DNA = 38 (QP at 0.4 δ/λ)
20 μ l 10X Sae
10 μ l $S\alpha$ I
10 μ l $B\beta$ II
152.5 μ l H₂O to 200 μ l

④ Digest pCG234 with $P\alpha$ A \bar{I} and $S\alpha$ I

7.5 μ l DNA = 38
20 μ l 10X Sae
10 μ l $S\alpha$ I
10 μ l $P\alpha$ A \bar{I}
152.5 μ l H₂O to 200 μ l

⑤ Digest pCG231 with EcoRI + tlenow

1.7 μ l DNA = 38 (prep at 1.8 δ/λ)
10 μ l 10X Eco
5 μ l EcoRI
83.3 μ l H₂O to 100 μ l

one hour.
+ 4 μ l 2 mM dNTP's
+ 2 μ l tlenow

30 min RT
65°C 5 min
bring to 200 μ l

Extract + dialyze overnight against 100 μ l TE

(2)

⑥ Digest PCR reaction #5 with SmaI (RT)

10 μ DNA
 20 μ 10 \times #3
 20 μ SmaI
 150 μ H₂O

Extracted all 5 digests 1X with phenol, precipitated.
 Missing in 10 \times #3

⑦ Digest PCR reaction #5 with EcoRI

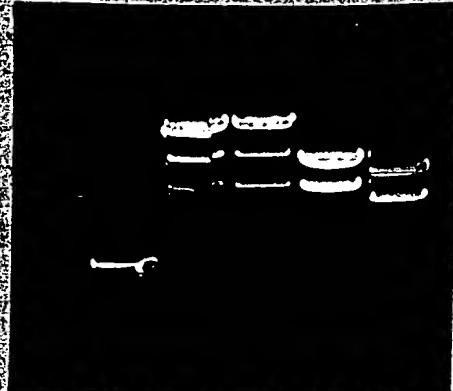
20 μ DNA
 5 μ 10 \times #3
 5 μ EcoRI
 20 μ H₂O

⑧ Add 10 μ of 10 \times MREB to reaction #5

20 μ DNA (from previous digest)
 5 μ 10 \times #3
 5 μ EcoRI
 20 μ H₂O

⑨ Digest PCR reaction #5 with KpnI

20 μ DNA (from previous digest)
 5 μ 10 \times #3
 5 μ KpnI
 20 μ H₂O



LMP gel

① PCR w/R Bgl II and PspA I

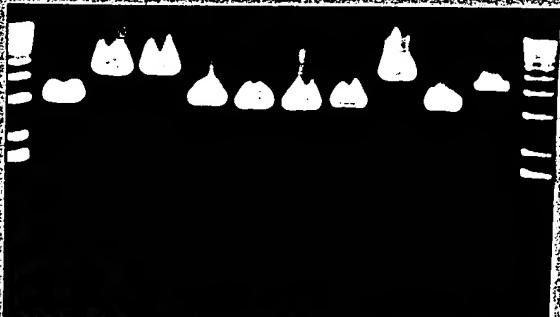
② ③ 234 w/R PspA I + Sph I

④ ⑤ 234 w/R Bgl II + Sph I

354 (1) 7.5% PCR gel
7.5% 234 .05%
5% 234 vector

354 (2) 5% Blank
5% 234 vector

Transformed into PG-1
GFP transformation cells

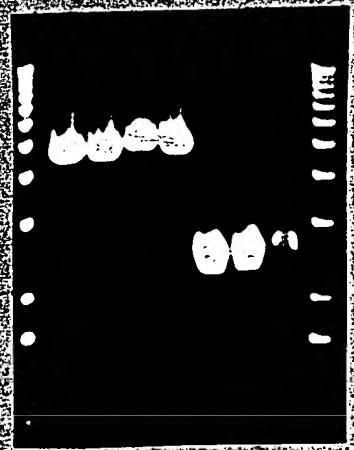


42-23 8% 100% 300%

R-Lab 2, 3 saved



further checking digest R-Lab 2, 3



K

304 R-Lab 2 & #3

Sma I

Bgl II

Bgl II + Hind III

234 (100ng) Bgl II + Hind III

21

DigiKit 304FM

304

Digest 100 μg DNA with SmaI and PstI (wout ~350 bp band)

0.6 μg DNA (15) Cat. 19811

5 μl 10X #4

4 μl SmaI

1 μl PstI

40 μl H₂O (to 50 μl)

15,35 → 0.6% CMA gel

Digest 100 μg DNA with SmaI and PstI (wout 1600 bp band)

0.5 μg DNA (8)

5 μl 10X #4

4 μl SmaI

1 μl PstI

40 μl H₂O (to 50 μl)

292 + neg (-)

5,35 → 0.6% gel

304

306

Ligations:

L1 5 μl 304 gel

L2 =

5 μl 306 gel

15 μl blank

10 μl 10X gel

=

1 μl ATP

=

1 μl T4 ligase

=

50 μl 10 mM Tris

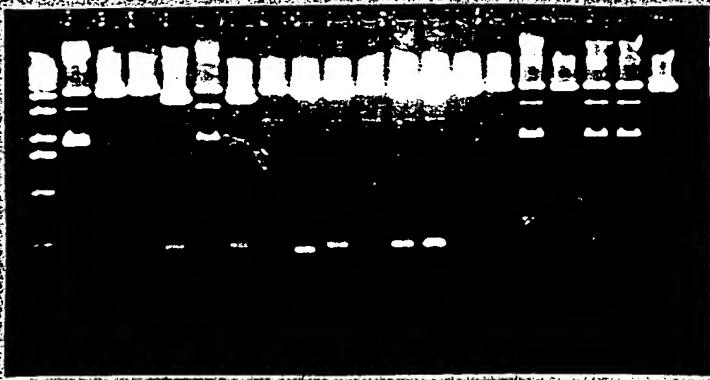
=

14 °C overn.

Gel bands from overnight

Digest were in 12 Row L 18 phret

Ropes digested with SmaI + BamHI #4, run on 1% gel



#1 used for GP

29



Do checking digests of 304 M

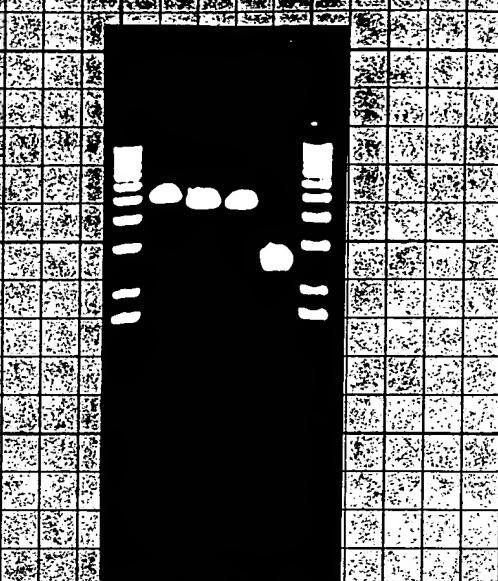
#3 ① Bgl II

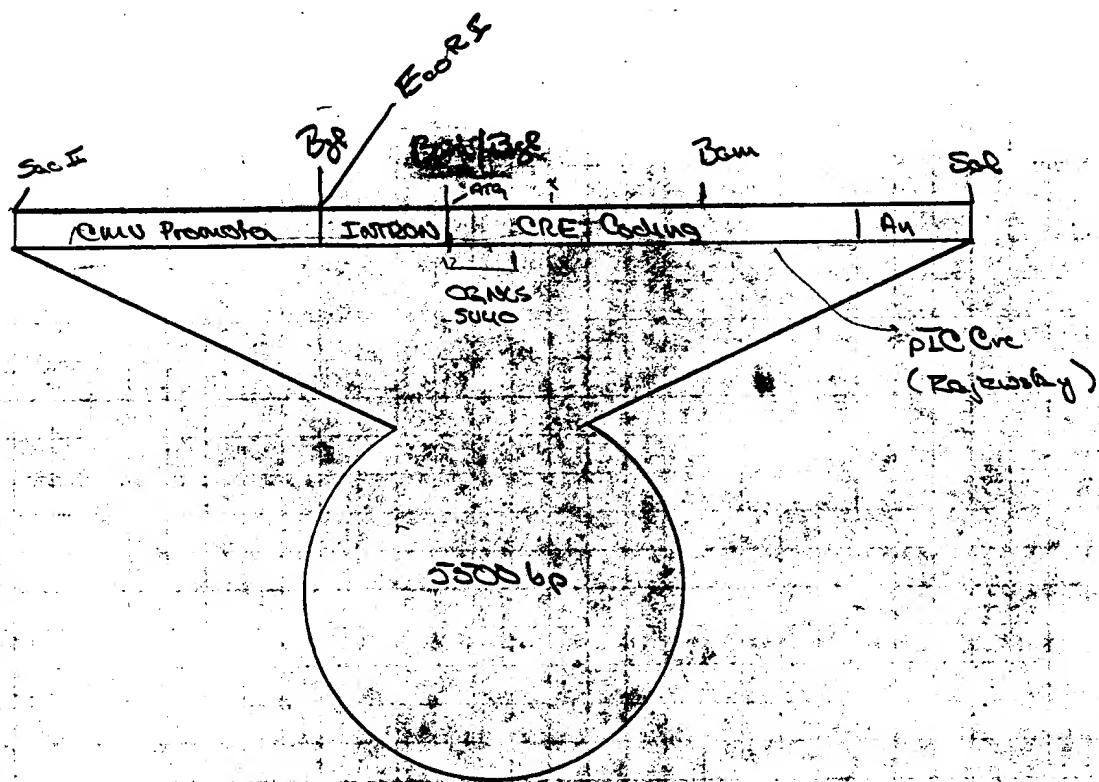
NC. #4 ② Sma I

Sal ③ Sal I

④ Bgl II + Sal I (Sal buffer)

DNA prep





Vector = pCC-19 ΔBB cut were BglII and SacI

Insert = pCC-239 cut with BglII and SacI

3/11

① Digest 234 with $Bgl\text{II}$:

DNA	-	30 λ
10X3	-	20 λ
$Bgl\text{II}$	-	20 λ
H ₂ O	-	130 λ

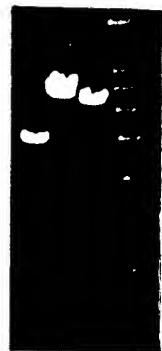
All three pieces digested one hour at 37°C. Then extracted with phenol/chloroform and precipitated.

② Digest 44 Δ B with $Bgl\text{II}$

DNA	-	25 λ (25 λ)
10X3	-	10 λ
$Bgl\text{II}$	-	7 λ
H ₂ O	-	80 λ

DNA spun down, washed

234 up in 30 λ
44 Δ B up in 25 λ } 1 λ each onto mini
 Δ B gel up in 30 λ



③ ~~Digest 44 Δ B with $Bgl\text{II}$~~

DNA	-	2 λ (3.2 λ)
10X3	-	10 λ
$Bgl\text{II}$	-	7 λ
H ₂ O	-	51 λ

SHOULD HAVE
CUT Δ B GEL WITH
BAM

SalI digest

234	DNA	30 λ
	H ₂ O	10 λ

DB	DNA	25 λ
	H ₂ O	15 λ

Δ B	DNA	30 λ
	H ₂ O	10 λ

all + 5 λ Sal buffer
5 λ SDE
one hour at 37°
2 λ → minigel
balance → UMA gel

From DB expect
3911 + 2011
From Δ B expect
3911 +



UMA gel (0.6% UMA in TAE)

- 1) 234, lower band excised
- 2) 44 Δ B, upper band excised.

Ligation

#1 15 λ 234 gel
5 λ 44 Δ B gel

+ 86 λ 10 mM Tris
12 λ 10X gel
1 λ ATP
1 λ ligase

#2 15 λ blunt gel
5 λ 44 Δ B gel

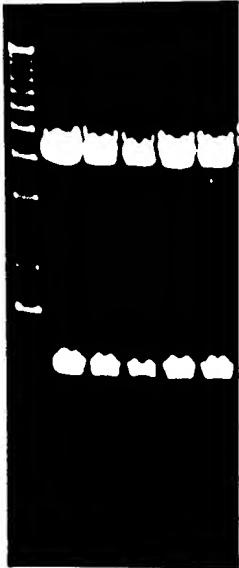
to 4°C @ 10PM

3/12

Transformations: St cooh mix into RS-1

Excellent transformation ratio obtained.

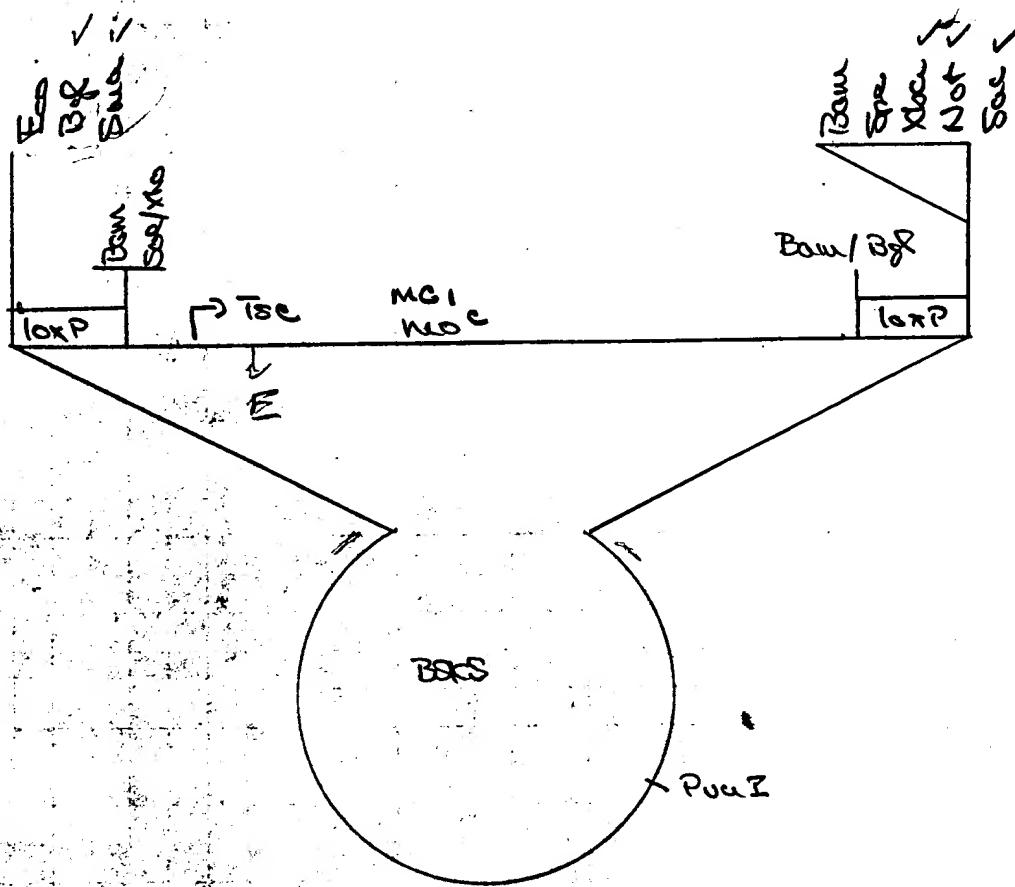
Colony picks for rapid lysates.



Std rapid lysates, $U_f = 50\lambda$, 1d for lysates
Sal + Bam Digest. All give expected patterns

#1 & 2 joined
#1 selected for Quagen

v = unique



PuuI to BglII from pOG249B = 1200 bp

PuuI to SeqI from pOG249S = 1659 bp

XhoI to BamHI from pMC1-HOC/BglA = 1133 bp

④ Digest 249B (R64) and 249S (R64+) with ScaI

20 μ DNA
10 λ ScaI
20 λ 10X #3
150 μ H₂O

5 λ → minigel
p/c extract
STOKE PPT with glycogen and NaOAc O/N
Wash pellet
Resuspend w/ 20 λ TE

⑤ Digest 249B with BglII

20 μ DNA
5 λ 10X #3
5 λ BglII
20 μ H₂O

5 λ → minigel
15 λ and 35 λ → 0.6% TAE
minigel,
Run 2 hours

⑥ Digest 249S with ScaI

20 μ DNA
5 λ 10X #3
5 λ ScaI
20 μ H₂O

249B ScaI/BglII 5 λ
249S ScaI/SalI 5 λ

LUMA gel



Excised bands.

249B: ~1200 bp band (lower)
249S: ~1700 bp band (upper)

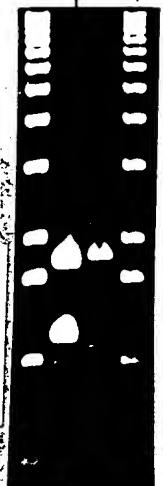
Ligation

277(1) 5 λ 249B gel
277(2) 249S gel
10 μ MCAmbo gel (7/11)

277(2) 5 λ 249B gel (-)
5 λ 249S gel (-)
10 μ blank gel

+ 86 λ (0 mM Tris 7.4
12 λ 10X gel ligation
1 λ 100 mM ATP
1 λ T4 ligase

-14° O/N -



5¹ digestion used to transform 50¹ AGS

no colonies

5¹ ligation \rightarrow 100¹ AG-1

Good colony ratio, low but ok #'s

plots left at 37° for 48 hr

12 "areas" picked into 5 ml LB + Amp.

Rapidly, 5 cultures grown up, each digested
with S1 Nuc and with Not I



I-S 5
I-S NOT

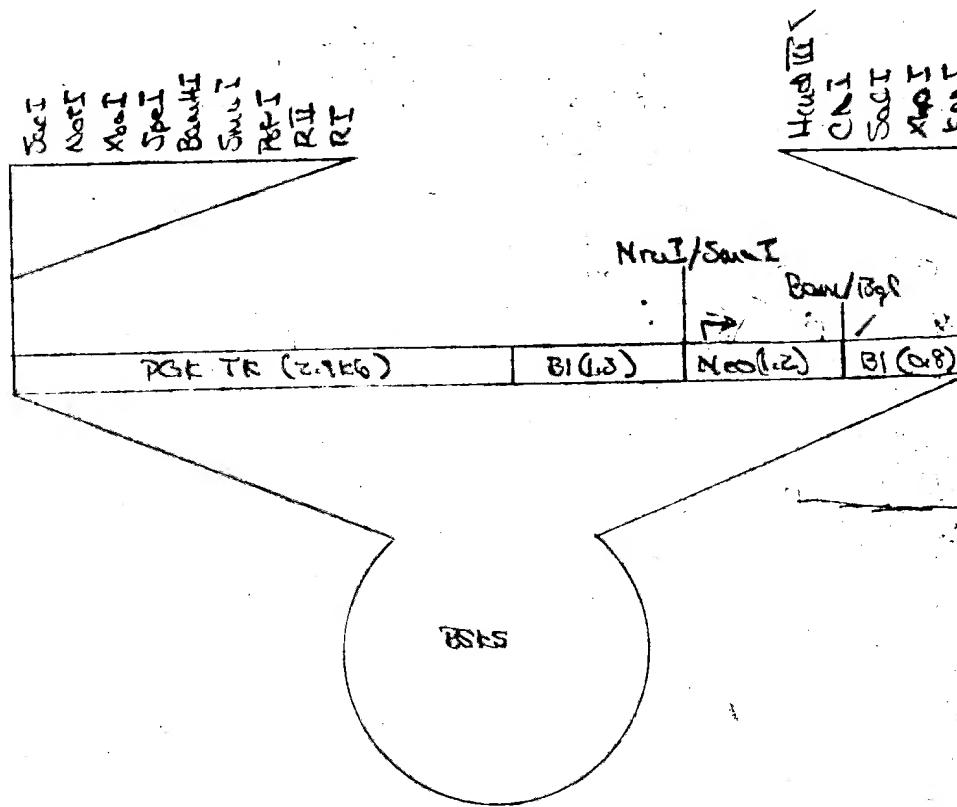
#1 & 2 look good

#1 used for Zeta transfection
test for recombination

#1 used for Qiagen prep.

LokP² NEO CASSETTE INTO PGK-TR + B1 Promoter

345



- 1) 336 NruI partial + HindIII
- 2) 336B BglII + HindIII
- 3) 277 BamHI partial + SacI

	4.2		1.2	1.3-7	EcoRI
	4.1			4.9	BamHI
1.0	2.4		5.5		SacI
					NheI
					XbaI
					KpnI
					HindIII

① Digest 3 λ POG 336 with Nru I

1.6 λ 336 (12/5/95 prep at 1.9 λ /1)
 20 μ l 10X Nru I
 5 μ l Nru I
 $\underline{173.4\mu\text{l H}_2\text{O}}$
 $\underline{200\lambda}$

② Digest 10 λ POG 313 with Nru I

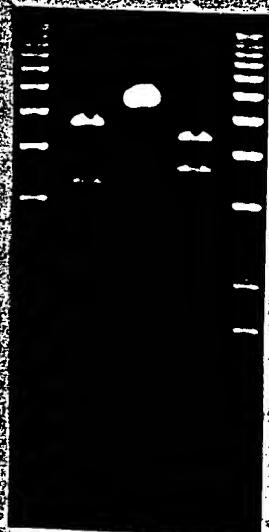
5.6 λ 313 (8/20 prep at 1.8 λ /1)
 20 μ l 10X Nru I
 $\underline{10\mu\text{l Nru I}}$
 $\underline{164.4\mu\text{l H}_2\text{O}}$
 $\underline{200\lambda}$

③ Digest 5 λ POG 336 with Eco RI

2.6 λ 336
 20 μ l 10X Eco RI
 $\underline{110\mu\text{l Eco RI}}$
 $\underline{167.4\mu\text{l H}_2\text{O}}$
 $\underline{200\lambda}$

One hour at 37°C

Run 7 λ ①, 2 λ ②, 4 λ ③ on a minigel
 P(C) PPT all three overnight.



336 Nru I 7 λ

313 Nru I - 2 λ

336 Eco RI 4 λ



336 (100ng) Nru I

Not I

Kpn I

342 (100ng) Kpn I

Nru I digest of 336
 showed unmaped
 site in Tc will
 need to do a search

343

① 10λ 336 cut w/ Not I $10\mu\text{l} = 200\mu\text{l}$ w/ 10λ enzyme

5.2λ DNA ($12.5/95$ prep at $1.9\gamma/\lambda$)

20λ 10X #3

10λ Not I

164.8λ H₂O

One hour at 37°C

$2\lambda \rightarrow$ minigel

PLC ppt, spin 70°C EtOH wash
resuspend in 20λ TE

$\geq 3\lambda$ Not I ✓

② Cut EcoRI digested 336 w/ Not I

$50\mu\text{l}$ of resuspended DNA, $50\mu\text{l}$ UT 5% enzyme

All 20λ DNA

(my mistake) 5λ 10X #3

5λ Not I

30λ H₂O

One hour at 37°C

$1.5\lambda \rightarrow$ minigel

③ Partial EcoRI digest of Nru-cut 313

All of resuspended DNA

$10\mu\text{l} = 200\mu\text{l}$

0.5λ EcoRI

3, 6, 12, 24, 45 minutes

run 4 λ aliquots on checking gel

20λ DNA

20λ 10X Eco

5λ EcoRI

159.5λ H₂O

④ Partial Nru I digest 336 (Not I) w/ Nru I

All of resuspended DNA

$10\mu\text{l} = 200\mu\text{l}$

0.5λ Nru I

3, 6, 12, 24, 45 minutes

run 4 λ aliquots on minigel

20λ DNA

20λ 10X Nru

0.5λ Nru I

159.5λ H₂O

336 Eco/Not I

0.6% LMPA/TAE gel

① 313 partial 6 min

30λ 336 EcoRI/Not I

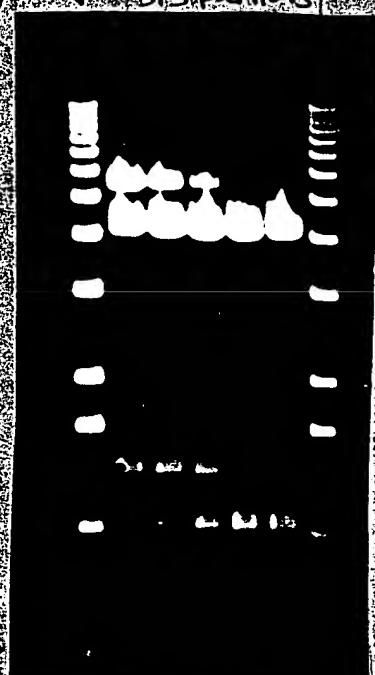
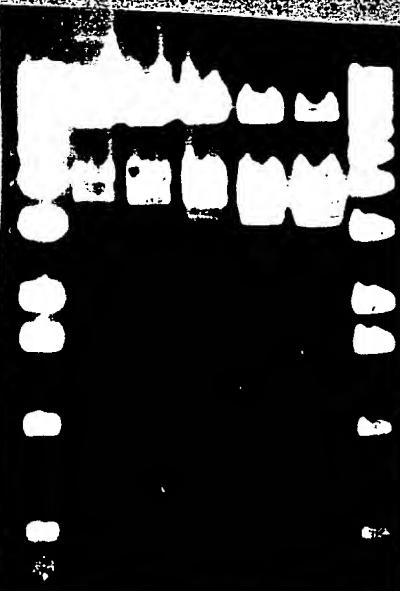
336 partial 3 min

6 min

12 min

24 min

45 min



Ladder

Min excised bands and
set up ligations

[] 94

Ligations L1 and L2 were transformed into
100 λ or lab stock AG1's.

Transformation plates show about equal numbers
of colonies on L1 and L2.

Picked 18 colonies for rapid's tomorrow.

Standard Rapid lysates done - all 18 grew up.

Set up EcoRI digests



6

20

① NruI partial digestion of 258 pOG336

$\cdot 13\lambda$ DNA (12/5/95 prep at 1.9 μ / λ)
 20λ 10X Nru
 0.5λ Nru I
 166.5λ H₂O
 $\underline{200\lambda}$

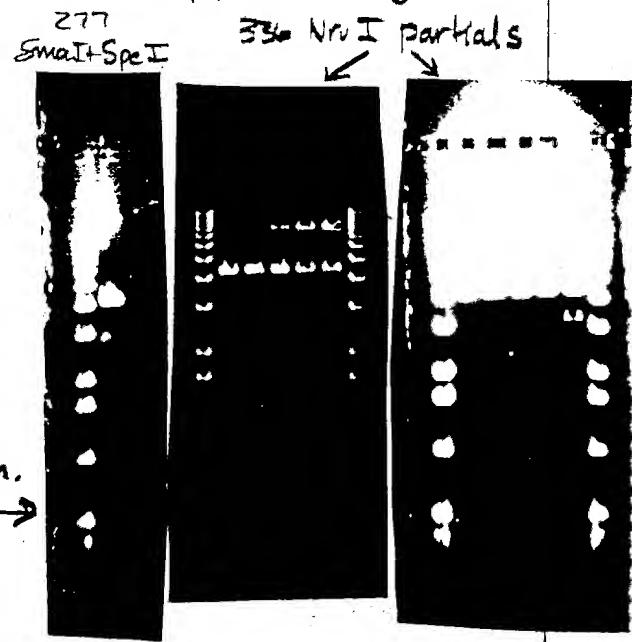
To 37°C, timepoints taken at 3, 6, 12, 24, and 45 minutes.
 $(40\lambda \rightarrow 4\lambda \text{ 250mM EDTA} \rightarrow \text{ice})$

1λ (125 ng) of each timepoint ran out on a minigel.
 P/C the rest of each timepoint and leave to ppt. overnight.

② SmaI + SpeI digest of pOG277

5λ DNA (7/30/94 prep at 1.8 μ / λ)
 20λ 10X #4
 5λ Sma I
 5λ Spe I
 165λ H₂O
 $\underline{200\lambda}$

Added 5 λ Sma I \rightarrow RT for 30 min.
 Added 5 λ Spe I \rightarrow 37°C for 30 min.
 $4\lambda \rightarrow \text{minigel}$ \longrightarrow
 P/C, ppt. overnight.



DNA was spun down for 30 min, washed with 70% EtOH,
 respun and resuspended in 20 λ TE.

Redo SpeI digest of SmaI + SpeI digested 277

20λ DNA
 20λ 10X #2
 20λ Spe I
 140λ H₂O
 $\underline{200\lambda}$

One hour at 37°C
 $4\lambda \rightarrow \text{minigel}$

$+ 8\lambda$ 2mM each dNTP
 4λ Klenow 30 min. at RT
 \quad 70°C for 5 min.
 P/C, ppt.

CIP 336 NruI cut DNA 45' time point

20 λ resuspended 336 DNA

5X 10 \times #2

3 λ CIP

22 λ H₂O

37°C 30', 15 λ 35 λ → 0.6% LMA gel

resuspend 277 SmaI + SpeI + Klenow DNA
in 20 λ TE onto one lane of LMA gel

Ligations

L 5X 334 gel 1
15 λ 277 gel 2
15 λ blank

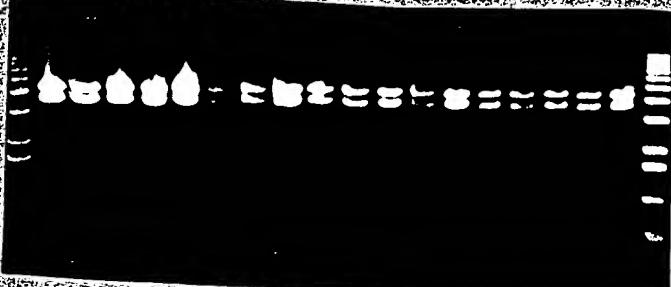
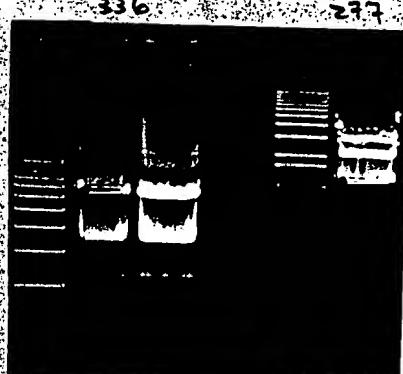
both 86 λ 10mM Tris
12 λ 10 \times gel buff
1 λ ATP 100mM
1 λ T4 ligase

14°C O/N

λ → 50 λ - Agar plate on LB + AMP plates

picked 18 colonies → 50 λ → F and GOD

QL #1-18 digest w/ EcoRI



did not get
wanted piece

- redo 343

5
22

① Nru I Partial digestion of 255 POG 36

13 λ DNA (12/5/95 prep at 1.9 λ / μ)20 λ 10X Nru I0.5 λ Nru I166.5 μ H₂O200 μ

To 37°C, timepoints taken at 3, 6, 12, 24 and 45 minutes.

(40 λ \rightarrow 4 λ 250 mM EDTA \rightarrow ice)1 λ (125 ng) of each timepoint ran out on a minigel.

P/lc the rest of each timepoint and leave to ppt. overnight.

336 Nru I
6.12.24.95

277 Spe I

② Spe I digest of POG 277

5 λ DNA (7/30/94 prep at 1.8/ λ)20 λ 10X # 2+10 λ Spe I165 μ H₂O200 μ

37°C for one hour

4 λ \rightarrow minigel

P/lc, ppt. overnight



The partial turned out more incomplete than we would like. It would be better if there were less of the supercoiled and relaxed circular DNA and more of the doubly-digested DNA, but as long as we do not overload the gel and as long as we run the digest out sufficiently far, we should be O.K.

The Spe I digest of 277 was not very complete - there was a substantial amount of supercoiled and relaxed circular DNA. Steve spun down the DNA, washed it, and redigested it with Spe I. He ran a small aliquot on a minigel and then cleaned up and precipitated the rest.

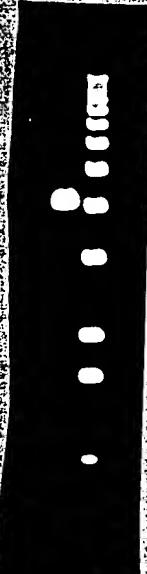
5

23

Redigestion of SpeI-cut 277 looks good

Spun down the SpeI-cut 277 and the 45 minute timepoint from the 336 NruI partial. Washed with 70% EtOH, re-spin, and resuspended both pellets in 20 λ TE.

Redigested
SpeI-cut
277



CIP 336 NruI

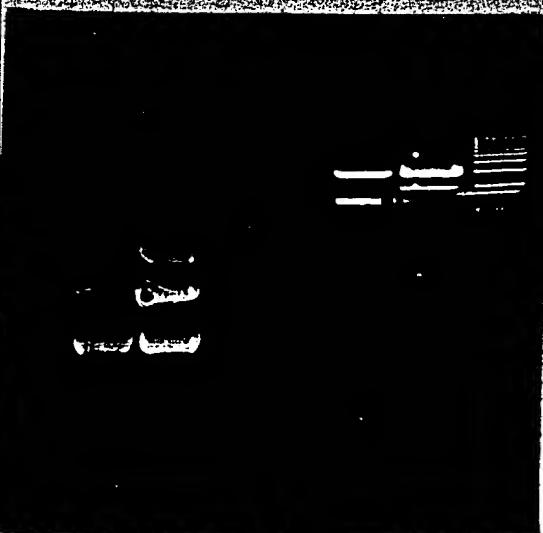
20 λ DNA	
5 λ 10X buffer	
2 λ CIP enzyme	
2.3 λ H ₂ O	
<u>50λ</u>	

30 min at 37°C

Load 15 & 30 λ onto a 0.69% LMA/TAE gel.
Run for a long time.
Want ~B6b band

0.69% LMA/TAE gel

- ① kb ladder
- ② 336 NruI (CIP) 15 λ
- ③ 30 λ
- ④ blank
- ⑤ blank
- ⑥ 277 SpeI (Klenow) SmaI 15 λ
- ⑦ 35 λ
- ⑧ kb ladder



Klenow of 277 SpeI

20 λ DNA	
2 λ 2mM dNTP's	
5 λ 10X buffer	
2 λ Klenow	
<u>21λ H₂O</u>	
<u>50λ</u>	

30 min at RT

10°C for 5 min.

P/C, Ppt, spin, wash with 70% EtOH,
re-cip, resuspend in 20 λ TE

SmaI digest of 277 SpeI (Klenow)

20 λ DNA	
5 λ 10X #4	
5 λ SmaI	
<u>20λ H₂O</u>	
<u>50λ</u>	

One hour at RT

1 λ → minigel

15 & 35 λ onto 0.69% LMA/TAE gel
(Run ~1 hour)

Want ~1200 bp band

Ligations

① 5 λ 336 gel
15 λ 277 gel

② 5 λ 336 gel
15 λ blank gel

BOTH:

86 λ 10mM Tris
12 λ 10X gel loading buffer
1 λ 100mM ATP
1 λ T4 DNA ligase

To 14°C O/N

⑤ 24

not many colonies on both L1, L2, plates
ratio good

Picked 24 colonies for Rapid



RL#1-24; 336. FwRI

I do not believe that
any of these are correct.

Construction

1. Bam-HI partial digest of 277

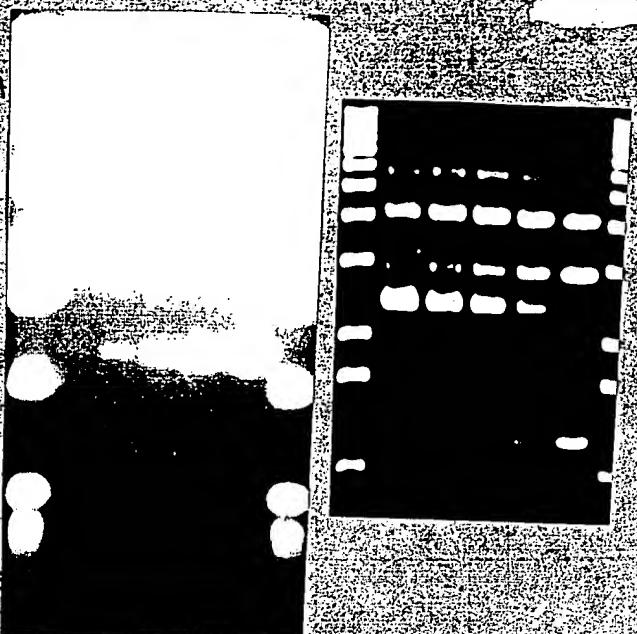
45 λ DNA (D87A-123095 QP)10 λ 10X #31 λ BamHI134 λ H₂O

37°C

time point 3, 6, 14, 24, 45'

1 λ of each \rightarrow mini geladd 4 λ 250mM EDTAadd 160 λ TE1 λ P/C ppt w/ glycogen

50% 70% wash

resuspend 12' time point in 20 λ TE

2. Sma complete of Bam partial 277

20 λ DNA4 λ 10X #34 λ SmaI12 λ H₂ORT 1 hr, 1 λ \rightarrow mini gel 1.5% \rightarrow 1% agar gel

← 277 Sma + Bam(P)

236 HindII + Mu(P)



3. Hind III complete of Nru I partial of 336

spin down 2

resuspend 12.96 NruI partial, 24' time point
in 20 λ TE (see 336 B notes)20 λ DNA4 λ 10X #24 λ HindIII12 λ H₂O37°C 1 hr, 1 λ \rightarrow mini gel1.5% \rightarrow 0.6% UMA TAE gel

4. Bgl II + Hind III digest of 336B

25 λ 336B RLH5 λ 10X #22.5 λ Bgl II2.5 λ HindIII1.5 λ H₂O37°C 1 hr, 1 λ \rightarrow mini gel1.5% \rightarrow 1% UMA gel

6/27

1% LMA gel



-277 Bam(P) + Sma
336 Nru(P) + Hind III

336B



336B Bgl II + Hind III

ligations:

L1 3 λ 336 Nru(P) + Hind III gel L2 : =
 S λ 277 Bam(P) + Sma I gel 10 λ 1.0% blank
 S λ 336B Bgl II + Hind III gel

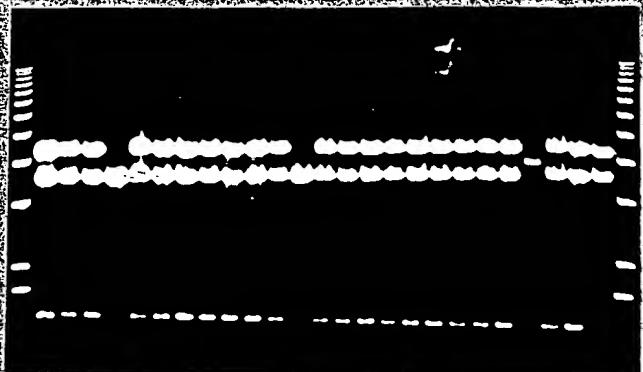
both 12 λ 10x gel
 1 λ 100mM ATP
 1 λ T4 l-gase
 9 λ 10mM Tris(4)

14°C O/N

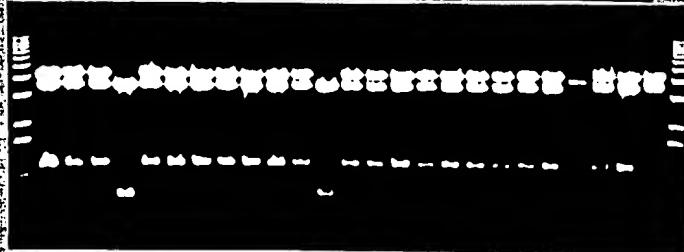
SAX-S50A Agar placed w/ AMP

Picked 24 colonies for rapids: 61, -2, ratio not good
 & low number of colonies on both plates

343 RL#1=24 control 336 w/ EcoRI



RL#1=3, 5=1, 13=21, 23=24 looks correct

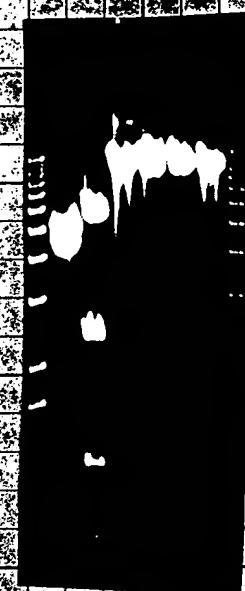


3

28

343 RL #1

Bam HI
Sac I
Not I
Sal I
Kpn I
Hind III



6

24

HOE ASSAY OF P2-S /304M | MOUSE TAIL DNA's

Standards

Conc.	Read #1	Read #2
0	0	12
100	104	109
200	193	201
300	312	302
500	482	480
750	746	729
1000	1000	968

Samples. 3A sample into 3ml assay buffer.

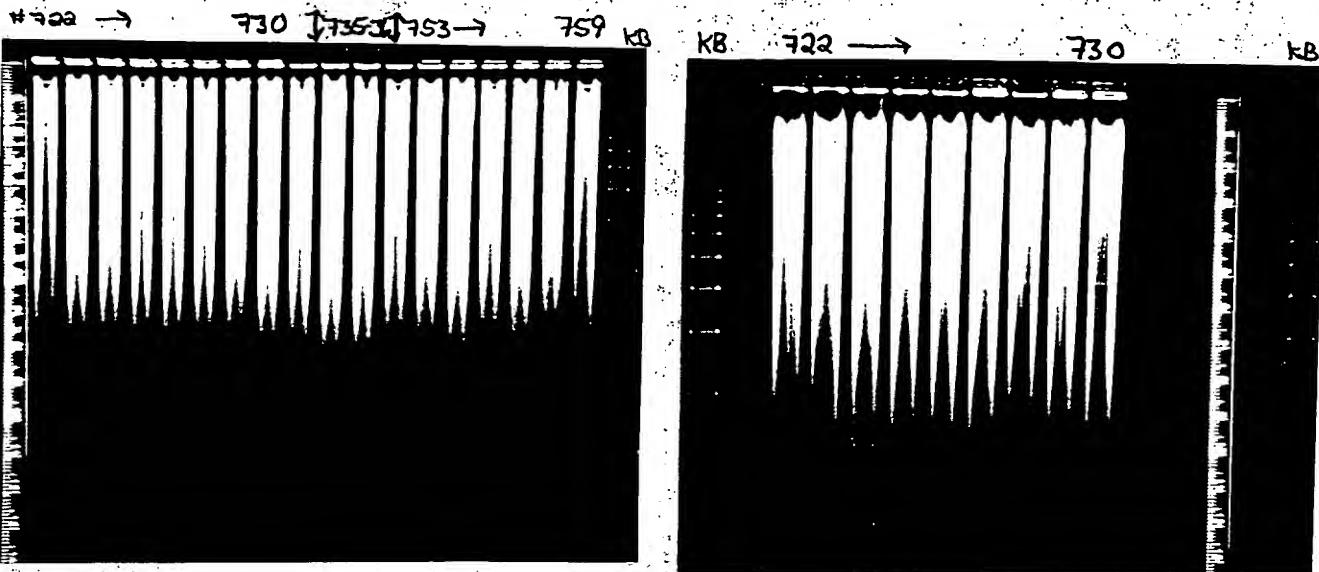
#	Cong. (ng/ λ)	$\lambda/10.4\mu$	10x(6A)	Enzyme (6A)	H ₂ O
722	342	29	BamHI	BamHI	19
723	360	28			20
724	444	23			25
725	397	25			23
726	431	23			26
727	295	34			14
728	450	22			26
729	378	27			21
730	448	23			25
735	791	15	*3	PstI	33
736	694	15			33
753	294	34	*3	PstI	14
754	540	19			29
755	415	24			24
756	520	19			29
757	317	32			16
758	547	18			30
759	434	23			25
722		29	*3	PstI	19
723		28			20
724		23			25
725		25			23
726		23			25
727		34			14
728		22			26
729		27			21
730		23			25

1:40 pm

8

30

SOUTHERN HYBRIDIZATION OF P2-S/304 M MOOSE TAIL DNA's



Pst I digests 121695A
P2-S P2-L probe 12-18-95



BamHI digests 121695B
304M Cre prob.



8

31

6ST 8SE tSE 1ST 2ST hSE

6ST

8SE

tSE

2ST

hSE

6ST

8SE

tSE

2ST

hSE

6ST

8SE

tSE

1ST

2ST

hSE

6ST

8SE

tSE

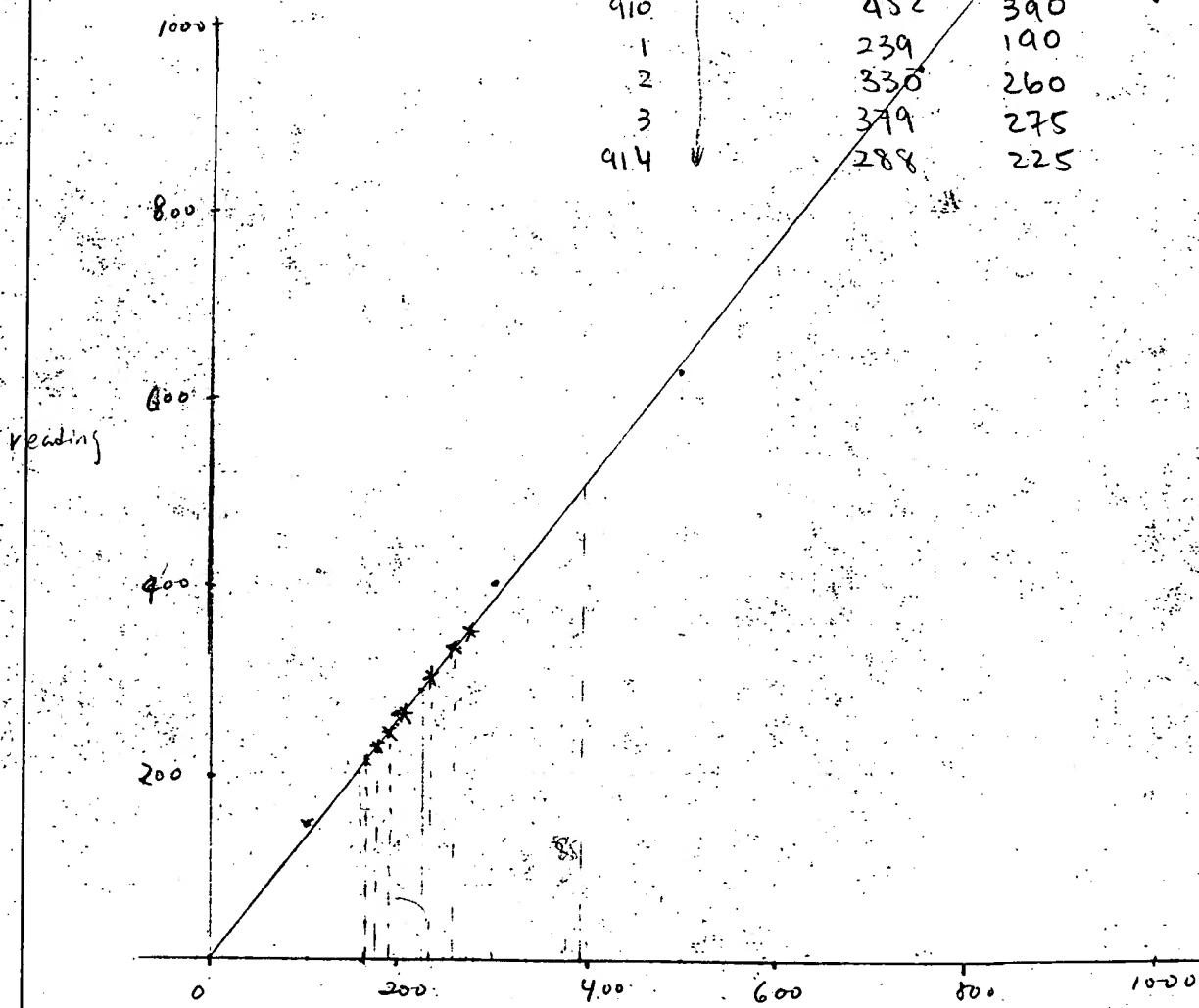
6ST

121695A
P2-5
P2-1 probe
24 hrs. exposure

(8) / 32

Mouse DNA Quantification

Standard	reading	Sample #	reading	acc. ng/μl
0	0	894*	346	280
100	145	895	225	175
200	267	6	264	200 mouse #894*-914
300	401	7 (6λ)	329	260 Embryo.
500	632	8	242	190
750	957	9	298	230
1000	1017	900	261	200
		1	243	190
		2	248	190
		903 (6λ)	211	160
		4 (9λ)	287	225
		5	298	230
		6	253	195
		7	295	230
		8 (18λ)	32	
		9 (9λ)	203	180
		910	452	390
		1	239	190
		2	330	260
		3	379	275
		914	288	225



• - standard
* - data

82

33

Southern

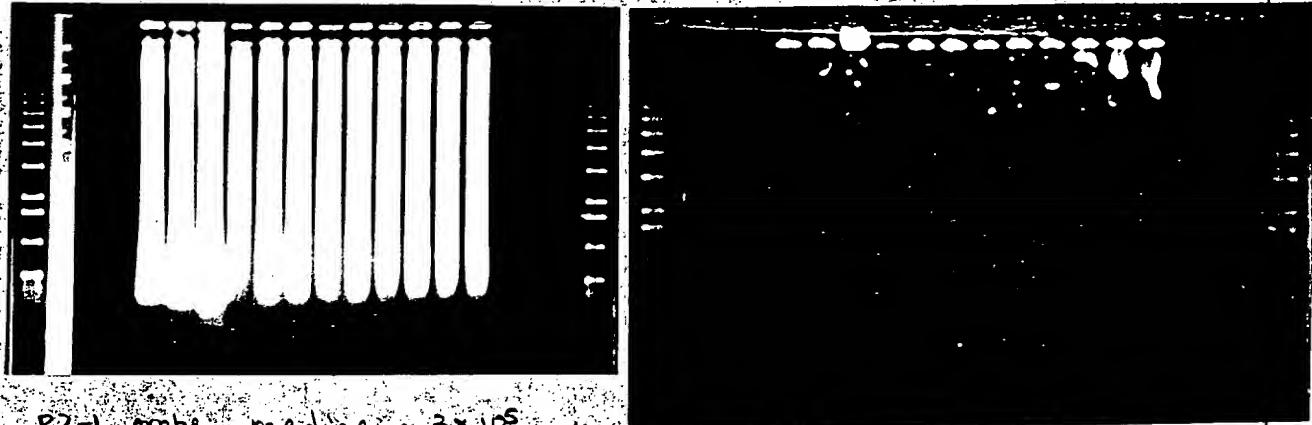
010696A

mouse Embryo DNA genotyping digest w/ PstI

lane #		DNA conc. ng/λ	DNA	H ₂ O	10x#3	EtBr
1	1 kb	-	λ	λ	λ	λ
2-4	-	-	-	-	-	-
5	TL	500	20	38	71	51
6	121495	600	17	41		
7	102595#5	212	47	17		
8	894*	280	36	22		
9	895	175	57	3		
10	896	200	50	8		
11	897	260	38	20		
12	898	190	54	4		
13	899	230	44	14		
14	900	200	50	8		
15	901	190	54	14		
16	902	190	54	4		
17-19	-	-	-	-	-	-
20	1 kb	-	-	-	-	-

22 V, 0.1N

standard transfer to pre-hyb for 8 hrs



P2-L probe reading $\sim 3 \times 10^5$
used all $\sim 100\lambda$

gel c

(8) / 34

Southern

010696

mouse Embryo DNA genotyping. Digest w/ Pst I

Lane #	DNA CONC	DNA λ	H ₂ O	10X #3	Enz.
		n.g./λ			

1 1Kb

2 TL

500

20

74

112

52

3 121495

600

17

77

4 102595 #5

212

47

47

5 903

90

0

6 904

7 905

8 906

9 907

10 909

11 910

12 911

13 912

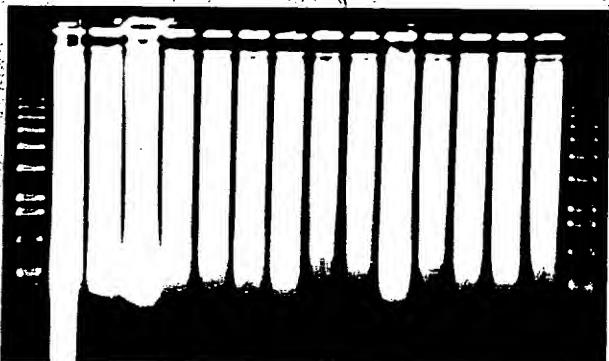
14 913

15 914

16 1Kb

22 V 0/N

Standard transfer to prehyb for 8 hrs



P2-1 probe reading $\approx 2 \times 10^5$
used all (n=100)



35

Blots of Embryos 894 to 914

- Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe . The signal was very strong and the A blot was very clean. The 910 sample of the B blot had an enormous signal in the 910 lane, which was the suspicious lane in the last probing with β -GAL. It would appear to me that somehow the sample that was loaded into this lane was contaminated with a Cre plasmid.
- The blot establishes that not all embryos showing the P2Br allele also contain the procre gene.
- The blots were discarded

Embryo #	Pol II	Pro Cre	β -GAL Stain
894	+ / +	+ / +	NEG
895	+ / +	+ / +	NEG
896	+ / P2Br	+ / +	POS
897	+ / P2Br	+ / 304	POS
898	+ / P2Br	+ / +	POS
899	+ / +	+ / 304	NEG
900	+ / +	+ / +	NEG
901	+ / +	+ / +	NEG
902	+ / P2Br	+ / 304	POS
903	+ / P2Br	+ / +	POS
904	+ / +	+ / 304	NEG
905	+ / P2Br	+ / +	POS
906	+ / P2Br	+ / +	POS
907	+ / P2Br	+ / +	POS
908	?	?	POS
909	+ / P2Br	?	POS
910	+ / P2Br	?	POS
911	+ / P2Br	+ / +	POS
912	+ / P2Br	+ / 304	POS
913	+ / +	+ / 304	NEG
914	+ / +	+ / 304	NEG
TOTAL			

6/36

ప్రాణ వైద్యతత్త్వ కులితోరి బ్రాహ్మ

P2-1 010696B
24 hr. exp.

2 125 25 200 2 2 5 2 900 900 900 900 900 900 900 900 900

(20) /38

53

441121

MS201

894

895

896

897

898

899

900

901

24 hr. cu

25 221 201 101 901 905 105 8
216 915 914

Mouse Embryo Genotyping-Southern Blots

Nicky developed the films of Min's blots that had been up to hyb since yesterday. These blots (010696 A and 010696B) contained samples of genomic DNA from embryos that were stained for β -GAL activity and which have been scored previously, see above. The blots were reasonably clean and the signal was strong.

Blot 010696A contained samples from embryos #'s 894* to 902*

- this blot contained the above samples and control DNA's from normal es cells, the original recombinant, and the Cre recombinant.
- None of the mouse samples showed the band expected for the original recombinant, they either showed only the wt allele or the wt allele and one copy of the Cre recombinant allele. The four animals showing the Cre recombinant allele were those that had been scored as β -GAL positive previously (#896, 897, 898, and 902).

Blot 010696B contained samples from embryos #'s 903 to 914 with the exception of 908, a sample that was lost during DNA extraction.

- 8 of 11 samples on the blot showed the pattern of hybridization anticipated for the Cre recombinant allele (903, 905, 6, 7, 9, 910, 911, 912). Three animals only showed the pattern associated with the wt allele (904, 913, 914).
- all of the animals that I had previously scored as β -GAL positive showed the Cre recombinant band on this blot.
- Sample 910 poses a real problem. In addition to the wt band and the Cre recombinant band this animal shows a band of the same size as the original recombinant and some additional apparently poorly digested DNA. The wt and p2br bands are present in approximately equimolar amounts, as would be expected, and they are of approximately the same intensity as the other samples. The additional p2bc band is stronger than either of these two, and would therefore have to represent an amplification of the unrecombined allele if it were to be real. It may well be that the expression of Cre in spermatids leads to some unusual rearrangements in addition to the anticipated rearrangements. Before getting too excited about this prospect it would be useful to extract DNA from the β -GAL-stained carcass if possible and to determine if this DNA shows the same pattern.

Friday,

Scoring embryos 903-914

- 903: I score this embryo as clearly positive, although the uniformity of staining is clearly disappointing. The most intense staining is in the GI tract and mouth structures, but there is clear staining of the somites as well. The vibrissal follicles are positive and the choroid plexus is also positive.
- 904: This embryo is entirely negative.
- 905: I score this animal positive with some reservations. The embryo was bisected coronally at the level of the forelimb. One side of the brain, viewed through the skin, is clearly positive. The other does not appear to be so. Some of the GI tract looks to be clearly positive, and the vibrissal follicles are faintly positive.
- 906: I score this embryo as positive in much the same sense as for 903. Some parts of the nervous system are reasonably well stained.
- 907: This embryo is very clearly positive. The CNS is well-stained as are the somites. Even in this embryo there is very considerable variation in the intensity of staining from region to region.
- 908: This embryo is positive in the 903 sense. The CNS is well, but variably, stained. The vibrissal barrels are faintly stained.
- 909: This embryo is clearly positive. The staining is intense in many places but it is blotchy.
- 910: This embryo is positive in the 903 sense.
- 911: This embryo is positive in the 903 sense.
- 912: This embryo is positive in the 903 sense, with some striking vibrissal staining and good forebrain staining.
- 913: This animal is clearly negative.
- 914: This animal is clearly negative.

894-902 Positives = 896, 897, 898, and 906

(8)/42

Friday,

Blots of Embryos 894 to 914

- Today Nicky developed 20 hour exposures of blots 010696 A and B after these had been probed with a Cre probe . The signal was very strong and the A blot was very clean. The 910 sample of the B blot had an enormous signal. This lane gave a puzzling result in the first probing of this blot with β -GAL; there were signals consistant with the other lanes that indicated that there was one wild-type allele and one P2Br allele, but there was additional signal. One part of this additional signal ran at about the same level as the P2Bc allele, another part of this signal was fairly high mw DNA. In the cre probing the whole lane is a smear - the kind of signal one sees in a blot of plasmid DNA when hundreds of ng of DNA are loaded. The only way to determine what is going on in this lane will be to run another gel with the newly extracted DNA from the rest of the embryo.
- The blot establishes that not all embryos showing the P2Br allele also contain the ProCre allele.
- The blots were discarded
- Overall, there were 21 embryos, 12 of the 19 that could be genotyped for RNA-PolII had the P2Br allele (none had p2bc), 7 of 18 that could be geneotyped for ProCre were positive, and 13 of 21 were positive for β -GAL. The correlation between β -GAL staining and the P2Br allele is 1:1.

Embryo #	Pol II	Pro Cre	β -GAL Stain
894	+/-	+/-	NEG
895	+/-	+/-	NEG
896	+/P2Br	+/-	POS
897	+/P2Br	+/304	POS
898	+/P2Br	+/-	POS
899	+/-	+/304	NEG
900	+/-	+/-	NEG
901	+/-	+/-	NEG
902	+/P2Br	+/304	POS
903	+/P2Br	+/-	POS
904	+/-	+/304	NEG
905	+/P2Br	+/-	POS
906	+/P2Br	+/-	POS
907	+/P2Br	+/-	POS
908	?	?	POS
909	+/P2Br	?	POS
910	+/P2Br	?	POS
911	+/P2Br	+/-	POS
912	+/P2Br	+/304	POS
913	+/-	+/304	NEG
914	+/-	+/304	NEG

New Blot of Embryos 894-902

- The first Southern blot (010696A) of these embryos looked pretty good - the samples are appropriate for attempting to make a publication Southern. Nicky poured a new 0.6% gel and did Pst I digests of the DNA's that were on that blot, and did an additional digest of 392 DNA. The expectation is that the somatic (tail)DNA of the 392 mouse, which sired this litter, will show the P2Bc allele, while the embryos he sired will show the P2Br allele. We did not have hoe assay results for the samples of 392 that we had previously prepared so Nicky read the OD 260 and we estimated that it would be high by about 1/3, so instead of running a nominal 10 μ g of DNA we ran a nominal 15 μ g of DNA - the latter should come close to a true 10 μ g of DNA because the sample is heavily contaminated with RNA.
- The gel was run overnight at 24 volts to minimize band smearing. On Wednesday it will be transferred and put up to prehyb overnight - it will be probed with P2-1 overnight on Thursday

③/44

$\text{NEO}^R + \text{TK}^R + \text{lacP} + \text{Cgal}$

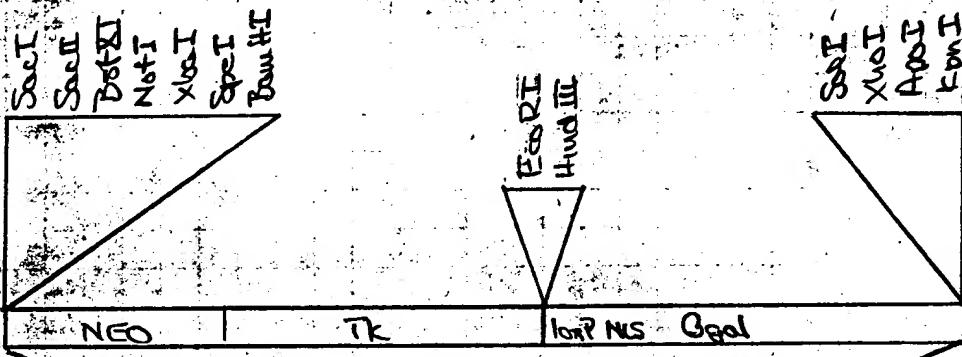
MUTANT NEO

235

235C Is Corrected Version

2900 3000 3100 3200 Sac Eco

3900 3000 3200



BEST II
2961

Vector = pGS227 cut with Hinc III and Sal I

Insert = pGS230 cut with Hinc III and Sal I

6 / 45

Digests for 235° and 239°

227
235
239

① Hind III digest of 227° RL #1

25 μ DNA
20 μ 10X TE
10 μ Hind III
145 μ H₂O

② Hind III digest pOSZ35

20 μ DNA 10 μ 10X 2.0M λ
20 μ 10X TE
10 μ Hind III
168 μ H₂O



③ Hind III digest of 239

1.5 μ DNA 12 μ 10X 3.3M λ
20 μ 10X TE
10 μ Hind III
168 μ H₂O

5) each digest → minigel
balance cleaned up and opt'd

1/11/94

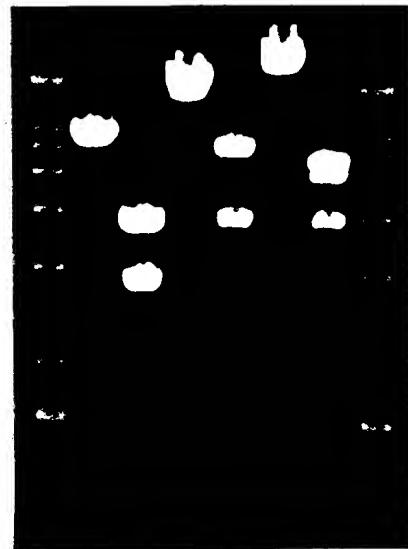
DNA spun down, resuspended in 20 μ , 1/1 into minigel.

Bam HI digests

Minigel

10 μ DNA
5 μ 10X Bam
5 μ Bam HI
80 μ H₂O

1/20 μ 227° Hind
1/10 μ 227° + Bam
1/20 μ 235 Hind
1/20 μ 235 + Bam
1/20 μ 239 Hind III
1/10 239 + Bam



Realized that I needed another strategy
for 239°

∴ Kpn I digest 239 cut with Hind III

10 μ DNA
5 μ 10X TE
5 μ Kpn I
20 μ H₂O

45 minutes at 37°C, then 2 μ → minigel

2 μ reserved on ice
2 μ for Bam HI digest
+5 μ 100 mM Tris
+5 μ 150 mM NaCl
2.5 μ 100 mM MgCl₂
+2.5 μ Bam HI
+1 μ 1.0 M

6/16

$$\begin{aligned} 150 \times 10^3 &= \\ 1500 & \\ \frac{5\text{M}}{1.5} &= 3.3\text{M} \end{aligned}$$

① Digest pOG230 with Hind III

have 20 μ g DNA from Gigaen prep of 230, from colony #4
OD 260/280 done on 1:100 dilution

SAMPLE	A320	A280	A260	280/260	260/280
	1.0000	0.0070	0.3048	0.5816	0.5182

Concentration of DNA = 2.98 μ g/ μ l. Will be isolating 3kb fragment from λ b plasmid. Digest about 5 μ g DNA

λ DNA λ \rightarrow minigel.
20 λ 10X #2 clean and ppt balance
10 λ Hind III
16 λ H₂O

② Digest pOG227 with Hind III

have Gigaen prep. from 101:93 = 1.68 μ g/ μ l

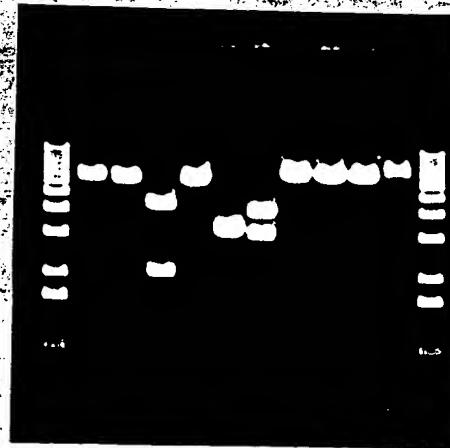
λ DNA λ \rightarrow minigel
20 λ 10X clean and ppt balance
10 λ Hind III
16 λ H₂O



Clock digests on plasmids, 100 ng each

#230 Kpn I (1)
 Hind III (1)
 Sac I (1)
 Sal I (S)
 Eco RI (E)

#227 Eco RI (E)
 Bam HI (B)
 Hind III (1)
 Sac I (S)
 Sma I (1)



DNA's spun down, washed, resuspended in 40 μl TE

Sce I digest (2^o) 230

DNA	20λ
10X	5λ
SDS	5λ
H ₂ O	20λ

Sce I digest (2^o) 227

DNA	20λ
10X	5λ
SDS	5λ
H ₂ O	20λ

Urea gel: 0.6% Urea in TAE



The two fragments of 230 were not as separated as I would have liked.

Ligation

#1	15λ 230 gel	#2	15λ blank gel
	5λ 227 gel		5λ 227 gel

5λ of each gel ligation was used to transform 50μl of each of 2 plates Agar. Standard plating.

8 colonies picked into 5ml LB + Amp (plates gave very good transformation ratio)

10/17/93

Std. repeats done. Pelds up in 50λ. If digested with EcoRI

10/18/93

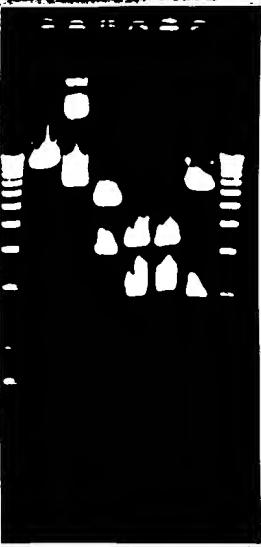
overloaded. 1, 2, 3, 4, 7 look to be correct.

#1 & 2 selected for Qiagen preps



Additional checks on 235 RC #1.

- Hind III
- XbaI
- Hind III + XbaI
- Sac I
- EcoRI
- SalI + BamHI



MCI uses Poly A cassette into pUC19

247

EGF I
SAC I
EGF II
SAC II
Bout II

SAC I / Xba I
EGF I
EGF II
SAC II
Bout III

← 100

pUC19
2686

50

MCI was PolyA → pUC19

247

① Digest MCI was with BamHI 5/28/90 prep is 5.75 μL

1 μl DNA
20 μl 10X Bam
10 μl BamHI
169 μl H₂O

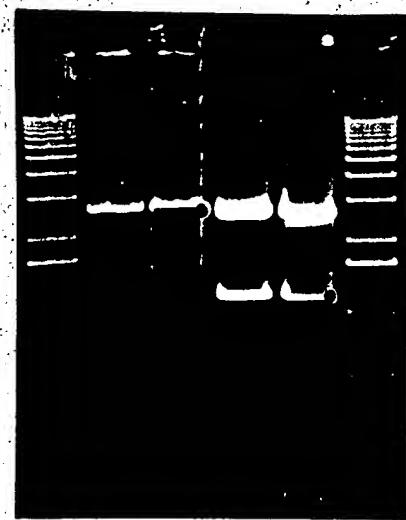


② Digest pUC19 with BamHI (TP prep at 0.9 μL)

5 μl DNA
20 μl 10X Bam
10 μl Bam
169 μl H₂O

1. 229
2. pUC
3. pMC-1

Both digests run one hour at 37°C.
Extracted and spot'd overnight.
Resuspended in 25 μl TE.
1 μl → minigel.



③ Digest Bam-MCI was with XbaI

15 μl DNA
5 μl 10X #2
5 μl XbaI
25 μl H₂O

④ Digest Bam-pUC with SceI

15 μl DNA
5 μl 10X #2
5 μl SceI
25 μl H₂O

Lanes gel: pUC19 15 μl
35 μl
MCI was 15 μl
35 μl

Bands excised and left in refrig overnight.

12/18 Standard ligations set up

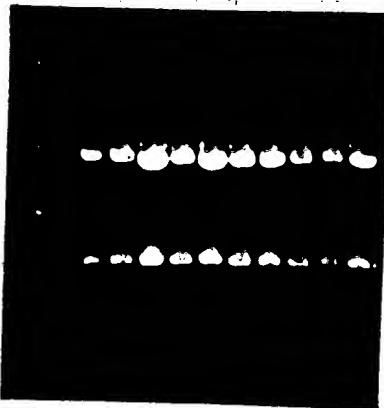
12/19 Transformed into AG-1

12/20 Good transformation ratio - 10 colonies picked.

51

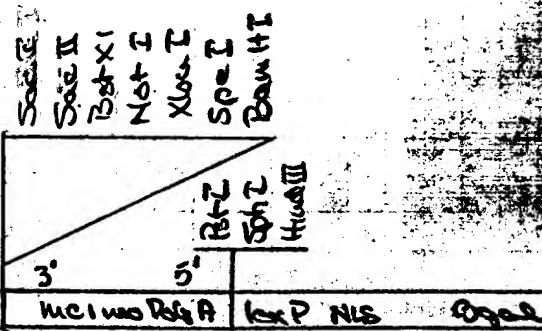
Rapids digested with EoRI.

All colonies look to be correct
#1 & #2 saved.



96560

320060



MCI neo Poly A cassette from pOG 247 (known to be wild-type)
Cre/LoxP and vector sequences from pOG 235

This plasmid is part of a replacement vector for the Pol II locus.

1/53

① Digest pGS247 with HindIII

2 λ DNA (12/27/93 prep at 3.58/ λ)
 20 μ l 10x#2
 10 μ l HindIII
 168 μ l H₂O



② Digest pGS235 with HindIII

2 λ DNA (10/19/93 prep at 2.68/ λ)
 20 μ l 10x#2
 10 μ l HindIII
 168 μ l H₂O

4 λ each digest loaded onto minigel
 DNA alcoholized up and precipitated
 resuspended in 20 μ l TE

look good



③ BamH1 & digest Z47 and Z35

20 μ l DNA
 0.1 BamH1
 5 λ Bam
 20 μ l H₂O

digested one hour
 2 λ → minigel

20 μ l of Z35 digest and 35 μ l of Z47 digest loaded onto minigel

Bands excised and standard ligations set up.

L1 5 λ Z35 gel
 (5 λ Z47 gel)
 12 μ l 10x gel lyg buff
 1 μ l 100 mM ATP
 1 μ l T4 ligase
 96 μ l 10 mM Tris

L2 =
 15 μ l blank
 =
 =
 =
 =



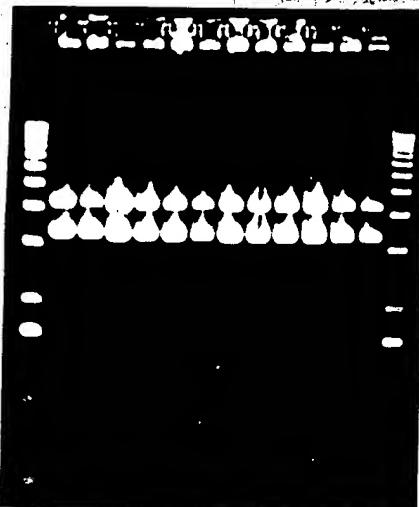
15/93 5 λ aliquots of ligations were used to transform 50 μ l aliquots of AS-1.

16/93 Excellent transformation rates obtained 710:1

b/54

Standard boiling rapid lysates. Up in 100?

11 aliquots digested with Eco RI



All samples yield fragments predicted for correct recombinant.

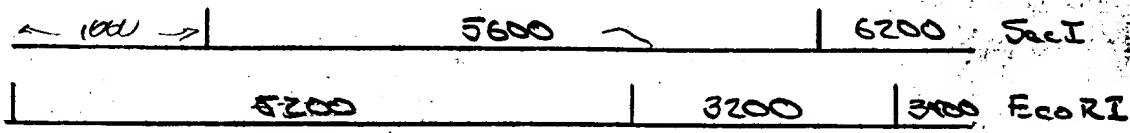
TK + Pol II plus Neo + β -gal for Replacement.

25*

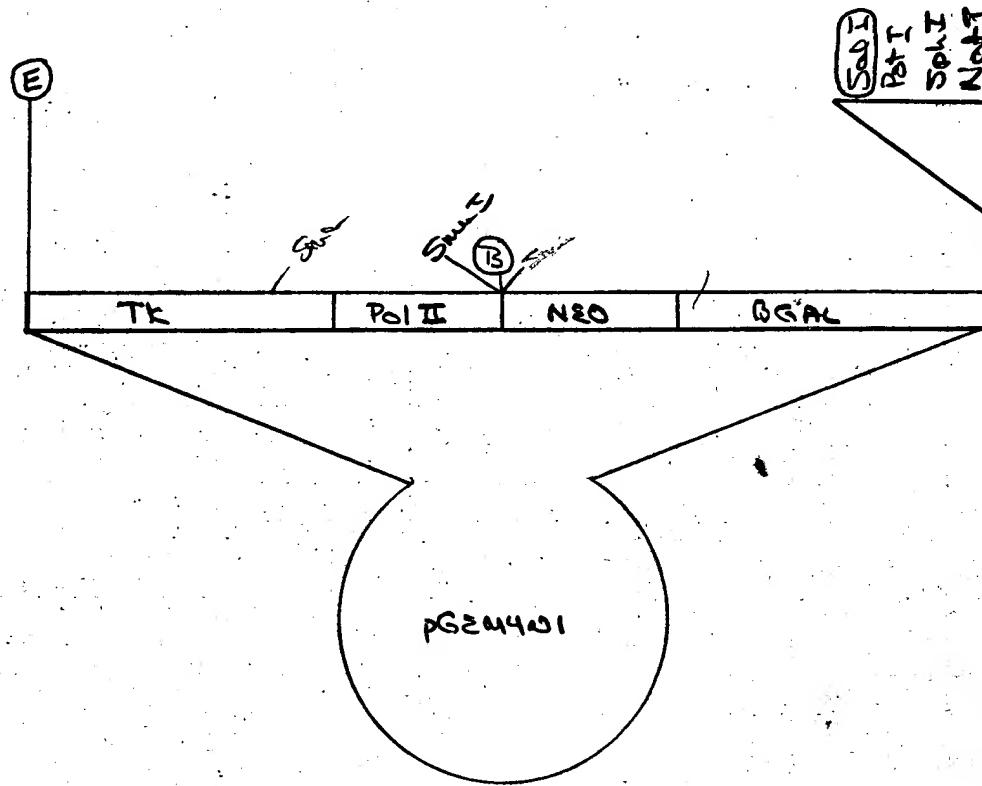
Hind III

Bam HI

Xba I



National Brand
42-182 100 SHEETS
Made in U.S.A.



Eco RI to Bam HI (4000bp) from 245 T.

Bam HI to Sac I (4800bp) from 250

Sac I + Eco RI (2900bp) from pGEM4zf1

156

Replacement

① EcoRI + BamHI digest 245

151 245 RL #1

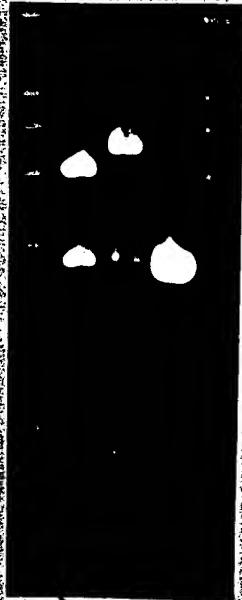
51 10X S2L

251 EcoRI

2.51 BamHI

301 H₂O

Minigel 5% each digest loaded on minigel



② BamHI + S2L digest 250

101 250 RL #1

51 10X S2L

31 S2L

21 BamHI

301 H₂O

③ Eco + S2L digest 0.5% CMA gel

11 DNA

51 10X S2L

31 S2L

21 EcoRI

381 H₂O

1/22/93 CMA gel 301 of 245, 250 digests and 10 and 201 of pGEM digest were loaded onto 0.5% CMA gel

Loaded Gel Incorrectly

Redo digests - as above. 5% each digest → minigel

201 pGEM, 301 245, 401 250 loaded on CMA gel.

pGEM 245 250

Ligations:

11 51 pGEM

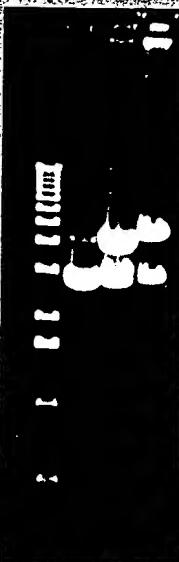
12 51 pGEM

7.51 245

151 blank

7.51 250

Other components standards

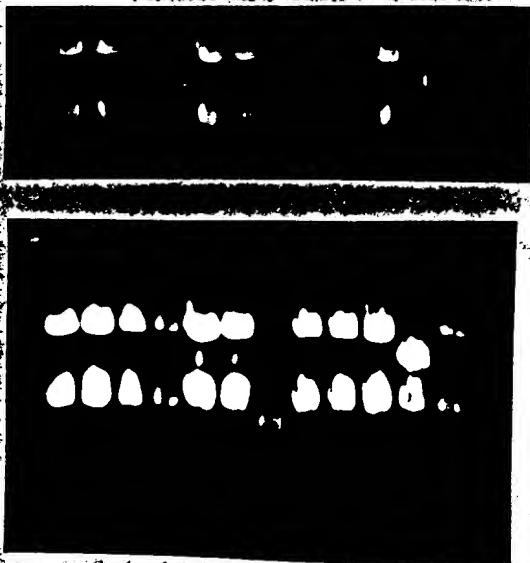


1/23 (1) each ligation → 1001 pGEM

1/24 excellent ratio obtained
12 colonies → pGEM

b/57

Std boiling temps - EcoRI digests:



All but 7 and 11 look correct

~3000 bp doublet cleaved in
#2

#2 and #6 saved.

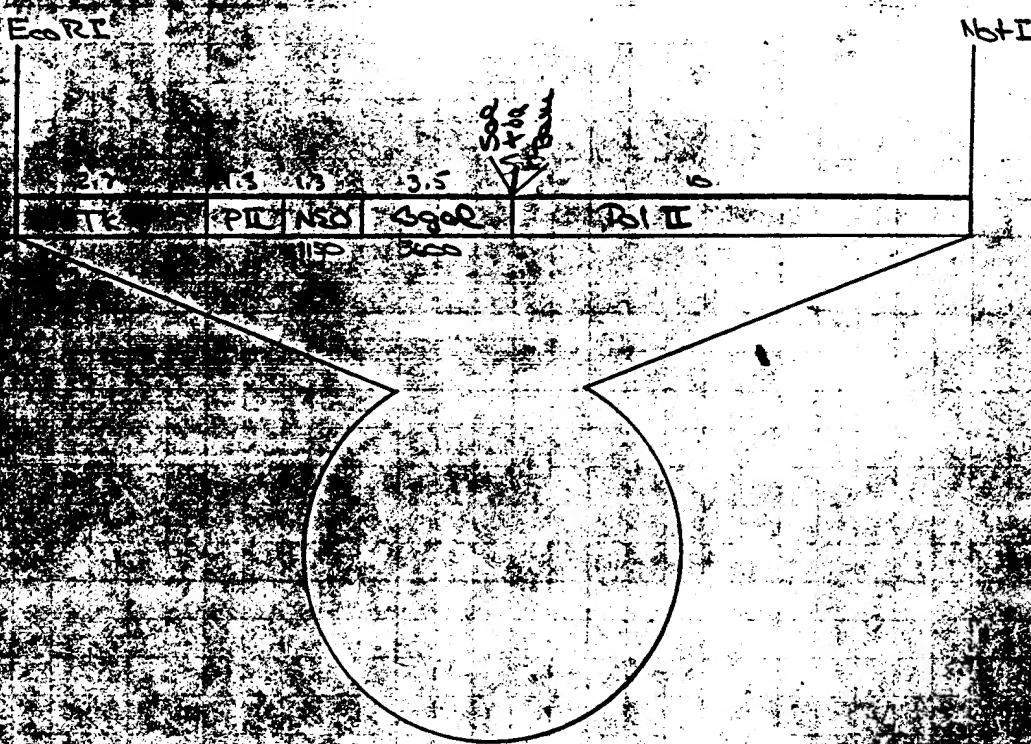
Numerous additional digests done for construction of 260-secondes

#2 scaled for Graven prep 1/26

Poll II - Bgal Replacement Vector

250

	6200	1600	2213	61	4117	7455	
500							
3500		3500	3503	8	4117	6100	5003
	5200		3600		13.5K6		EcoRI
9.5			4.0	2.4	5862		HindIII
			4.7		5768	14	BamHI



Vector = Z54 cut with SphI and NotI. Includes T5, ColE1, 100, Bgal, (α 114th)

Inserf = Z55N cut with SphI and NotI. Includes α 114th of BsiII gene and sequence

b/59

Further digests on 254 RL#2

2	ECORI	(Eco)
4	BamHI	(Bam)
1	XbaI	(#2)
3	Eco + Bam	(#3)
5	NotI	(#3)
6	SacI	(Sac)
7	Sac + Bam	(Sac)



? spontaneous digest 254

- 1) 254 RL#2
- 5) 10% Sac
- 3) 50% I
- 2) 10% I
- 30) 1% H₂O

Processive digest 255N

- 1) 255N prep 1/19/94
- =
- =
- =

39 x 4% agarose

- ① Add all components except NotI
- ② Incubate 5h → 37°
- ③ Add NotI

EMMA gel

- 1) 10) 254
- 2) 35) 254
- 3) 40) 255N
- 4) 10) 255N



- 1) 10) 254 - Not + Sac
- 1) 10) " Sac only
- 1) 10) 255N Not + Sac
- 1) 10) 255N Sac only

It appears that the SacI digest of 254 may be incomplete.

EMMA gel - want to use about 2x the amount of 255N to get good concentration of fragments.

New Digests

201 254 RL + 2 (new dilution to compare to previous digest)

201 10x SCD

151 SCD II

155 1450

1/3,01 of each digest

31 255N prep 1/19/94

201 10x SCD

101 SCD I

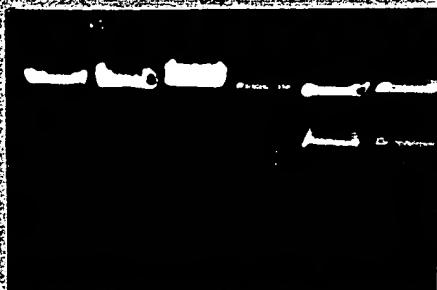
(61) H₂O

digests cleaned up and noted overnight.
resuspended in 20x TE.

1/27/94

- 2.6 μl each 10x aliquot of 10° digest was
digested with Not I in VT = 40°, with
4μl enzyme total

- after one hour digestion each digest was
loaded onto a 0.6% TAE (MNA gel),
run at 30V



Indicated bands excised (photo w/
out excision)

Ligation:

L 31 254 gel
71 255N gel

L2 31 254
71 255N

1/28/94 10x each ligation was used to
transform (2) aliquots of FC I + 61 10x
+ 1x A.T.P.
+ 1x ligase
+ 40 μl 0.5M TMS

1/29/94 Obtained approximately 10.1 L1:L2 ratio

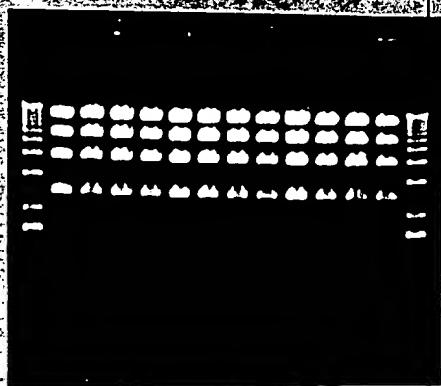
1/30/94 12 colonies picked into LB + Amp.

1/31/94 Std vectors digested with Hind III

All look correct

1/2 saved

1 restreaked, streaked,



61

36 ♂ ? Southern not clear sac p2-S F₀ on ♀ x C57BL/6

10 b 6-24-95

all Agouti

37 ♂ +/-

38 ♀ +/-

39 ♀ +/-

40 ♀ +/-

41 ♀ +/+ sac

42 ♂ +/+

43 ♀ +/+

44 ♀ +/+

45 ♂ +/+

C57BL/6				
46	♀	+/-	p2-SF, or + 7x C57BL/6 ♀	
47	♂	+/-	7 b tails	
48	♀	+/-	All Agouti	
→ 49	♂	+/-	mouse with tail punch sliced 439-453 0/13 positive	
50	♀	+/-		
51	♀	+/-	sac	
52	♀	+/-		
✓ 53	♀	+/-	sac	304 M F.
✓ 54	♀	+/-	sac	6 b
✓ 55	♀	+/-	sac	tails
✓ 56	♂	sac	8/15 +/+	1/6
✓ 57	♂	sac	8/15 +/+	
✓ 58	♀	+/-	304 M	

7/8/63

59	♂	+/-	SAC	P2-S F ₁
✓ 60	♀	+/-	SAC	P2-S F ₀ ♂+♀ x C57BL/6 ♀
61	♂	+/-		7 b
62	♀	+/-	SAC	(9 total in litter, 2 died)
63	♀	+/-	SAC	All agouti
64	♂	+/-	SAC	
✓ 65	♀	+/-	SAC	
66	♀	+/-	A/	SAC P2-S F ₁
67	♂	+/-	A/	P2-S F ₀ ♂+♀ x C57BL/6 ♀
			"	2 b tails
68	♂			304 M F ₀ (129 SV)
69	♂			3 b
70	♀		+/304M	tails
71	♀	B/- A/-	+/304M	SAC 12/12 304 M F ₀
72	♂	-/- -/- c/c	+/304M	no good as sac SAC 12/11/95 9 b
73	♂	B/- A/-	SAC	tails
74	♂	-/- -/- c/c	SAC	
75	♂	B/- A/-	+/304M	SAC
76	♂	-/- -/- c/c	SAC	
77	♀	-/- -/- c/c	SAC	
✓ 78	♂	b/b a/a	+/304M	(high copy number) SAC
✓ 79	♀	b/b A/-	SAC	

1 ⑧/4

80	♀ B - A -	Sac	304 M F ₀
81	♂ B - A -	↓	10 b
82	♀ -/- c/c	↓	tails
83	♂ B - a/a	+/304M ✓ Sac	
84	♂ B - A -	Sac	
85	♂ -/- c/c	↓	
86	♂ B - A -	? +/304M (signd low) Sac & 102	
87	♂ b/b a/a	Sac	
88	♀ B - A -	↓	
89	♀ B - A -	↓	

3-7-95

90	♀ B - A -	304 M F ₀	
91	♂ -/- c/c	15 b	
92	♂ B - A -		
93	♀ -/- c/c		
94	♂ B - A -		
95	♂ b/b A -		
96	♀ B - A -	+/304M ✓ Sac (high copy number)	
97	♂ B - A -		
98	♂ -/- c/c		
99	♀ B - a/a	+/304M ✓	
100	♂ B - A -		
01	♂ B - a/a		
02	♂ b/b A -		
03	♀ B - A -	+/304M ✓ (high copy #)	
04	♂ b/b a/a		

718/65

884

5

+/+
+/+

PZ-5

885

7

+/+

8

+ (PZ-5)
+ (PZ-5)

9

+ (PZ-5)

890

1

+ (PZ-5)

2

+/+

3

+/+

4

+/+

895 a/a

6

Scd

7

Scd

8

Scd

900

1

2

3 d.?

4

+/+
+/+

304

+/+

↙

+ (304)

+ (304)

+/+

304MLK PZ-5

♀ 547 (+304MLK, B)

♂ 503 (+ (PZ-5))

plug

harvest

blast 123095A

PBX-118

F₀ ♀ 243 x F₀ ♂ 241

96

66

	P25		
894*	+/+	-	304 - P2B
5*	+/+	-	CBGF, ♀
6*	+ P2Br	-	plugged by
7*	+ P2Br	+ 304	304M/P2B o? 392
8*	+ P2Br	-	harvested
9*	+ P2Br	+ 304	Blot 010685 A
900*	+/+	-	
17	+/+	-	
2	+ P2Br	+ 304	
903	+ P2Br		304 - P2B
4	+ +	+ 304	CBGF, ♀
5	+ P2Br	-	plugged
6	+ P2Br	-	by 304M/P2B o? 519
7	+ P2Br	-	harvested
8	+ P2Br	-	bolt 010685B
9	+ P2Br	-	bolt 011796
910	+ P2Br < ???	-	
1	+ P2Br	-	
2	+ P2Br	+ 304	
3	+/+	+ 304	
4	+/+	+ 304	

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